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THE AGAR/GEL PRECIPITIN TECHNIQUE IN ANTHRAX ANTIBODY DETERMINATIONS

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THE AGAR/GEL PRECIPTIN TECHNIQUE IN ANTHRAX

ANTIBODY DETERMINATIONS

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ACKNOWLEDGMENTS

ABSTRACT

A modification of the agar/gel precipitin inhibition technique of Thorne and Belton[1] for detecting anthrax antibodies reduces inconsistency of visually determined end points on the same sera observed by different technicians.

Determination of the minimum reacting concentrations of the anthrax antigen and antibody reagents, modifications of the visualization apparatus, methods for combining reagents, and length of incubation periods contributes to the ease of the end point determinations and the uniformity of results.

When compared with the previous technique, the modified procedure is less time-consuming and retains satisfactory reproducibility, simplicity, specificity, and sensitivity.

I. INTRODUCTION

Thorne and Belton[1] applied a modification of the technique of Ouchterlony, employing precipitin-inhibition and agar/gel diffusion, for determining the serological pattern following anthrax infection or immunization. The test proved equally valuable for the assay of anthrax antigen preparations. Employing the technique, Norman et al[2] determined serological responses to anthrax immunization in employees of a goat hair processing mill, but encountered considerable difficulty in obtaining uniform and consistent agreement among visually determined end points when different technicians evaluated the same sera.

This report deals with several modifications that contribute to the ease of end-point determination without detracting from the desirable sensitivity or reproducibility of the test.

II. MATERIALS AND METHODS

A. PREPARATION OF CULTURE MEDIUM FOR PRODUCTION OF ANTHRAX ANTIGEN

The method was adapted from the procedure as published by Strange and Thorne.[3] Stock solutions of each of the following ingredients were prepared in desired volumes and stored at 4°C for periods up to four months:

Ingredient	Concentration mg/100 ml	Treatment
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	73.5	Dissolved in 100 milliliters of distilled water.
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	98.6	Dissolved in 100 milliliters of distilled water.
Manganese sulfate ($MnSO_4 \cdot H_2O$)	8.5	Dissolved in 100 milliliters of distilled water.
Monopotassium phosphate (KH_2PO_4)	6800.0	Dissolved in 100 milliliters of distilled water.
Dipotassium phosphate (K_2HPO_4)	8710.0	Dissolved in 100 milliliters of distilled water.
Thiamine hydrochloride	5.0	Dissolved in 100 milliliters of distilled water.

Ingredient	Concentration mg/100 ml	Treatment
Adenine sulfate	21.0	Both adenine sulfate and uracil were dispensed into
Uracil	14.0	a 100-milliliter graduated cylinder containing 50 milliliters of distilled water. Ten normal sodium hydroxide was added by drops with constant stirring until solution was effected. Final volume was brought to 100 milliliters with distilled water.
1-tryptophane	520.0	1-tryptophane was dissolved in 20.0 milliliters of distilled
1-cystine	120.0	water to which had been added six milliliters of 6 N
Glycine	150.0	hydrochloric acid. Twenty milliliters of distilled water were added and the 1-cystine and glycine suspended in the solution. The suspension was vigorously agitated until solution was accomplished. Final volume was brought to 100 milliliters with distilled water.

The medium was prepared by placing 10 milliliters of each of the stock solutions in a 1000-milliliter graduated cylinder. Five hundred milliliters of distilled water and 3.6 grams of casamino acid medium (Difco) were added and the mixture shaken until solution was effected. Final volume was brought to 1000 milliliters with distilled water and the pH adjusted to 6.9. Four-hundred and fifty milliliter volumes were dispensed into three-liter Fernbach flasks. Two milliliters of a stock suspension of Norit A* (3.75 grams in 100 milliliters of distilled water) were added to each of the 450-milliliter portions in the Fernbach flasks and the resulting mixture was autoclaved at 15 pounds pressure at 121°C for 15 minutes. The sterilized medium was cooled to room temperature and to each flask was added 44 milliliters of 9 per cent sodium bicarbonate and 5 milliliters of 20 per cent glucose, both of which had previously been sterilized by filtration through ultra-fine fritted glass filters.

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* Norit A (vegetable charcoal): Matheson, Coleman and Bell, Inc., Norwood, Ohio.

B. PREPARATION OF ANTHRAX ANTIGEN

Each Fernbach flask, containing approximately 500 milliliters of the prepared medium, was inoculated with 2×10^8 spores of the Sterne strain of <u>Bacillus</u> <u>anthracis</u> and incubated at 37°C for 27 hours.

It is emphasized that all subsequent steps in the processing of the culture after incubation must be conducted in a room maintained at 4°C. The Norit A suspension was removed from the resultant culture by centrifugation at 1500 rpm for ten minutes. Organisms were then removed from the supernate by filtration through ultrafine, tubular, fritted glass filters (Pyrex). At this point the potency of the crude antigen filtrate was estimated by an agar diffusion method to be described; titers of 1:4 or greater were acceptable.

The crude culture filtrate was saturated with ammonium sulfate (760 grams per liter of filtrate) and the mixture allowed to stand at 4°C for 16 to 20 hours, thus "salting out" a reddish-brown precipitate containing the active antigen fraction.

The precipitates from two of the original culture flasks were separated by filtration through a Buchner funnel fitted with #602 Schleicher & Schuell filter paper and then washed with approximately 10 milliliters of 80 per cent saturated ammonium sulfate.

The filter paper with the adherent precipitate was subsequently macerated in 50.0 milliliters of 0.05M tris (hydroxymethyl) aminomethane at pH 8.0. Filter paper fragments were removed from the reddish-brown suspension by filtration through Whatman #1 filter paper on a Buchner funnel.

Ten-milliliter samples of the resultant solution were dispensed into sterile 50.0-milliliter vaccine-type bottles fitted with rubber stoppers, and 0.5 milliliter of normal horse serum was added to each vial under aseptic conditions. Contents were then frozen in an acetone/dry ice bath and immediately lyophilized.

A single vial was selected at random from each lot, the contents were reconstituted with 10 milliliters of sterile distilled water, and antigenic potency was ascertained by an agar-gel diffusion method to be described; titers of 1:16 or greater were acceptable. Specificity of the antigen preparation was checked against known positive and negative anthrax antisera. Reconstituted antigen preparations were stored for eight months at -15°C without loss of potency.

C. PREPARATION OF HYPERIMMUNE SERUM

Anthrax antigen was prepared by seeding the Sterne strain of <u>Bacillus</u> <u>anthracis</u> on a sporogenic medium composed of 8.0 grams of nutrient broth (Difco), 5.0 grams of sodium chloride, 2.0 grams of glucose, and 25.0 grams of agar per liter of distilled water with an adjusted final pH of 7.3; suitable volumes of the medium were dispensed in Roux bottles. Cultures were incubated for 48 to 72 hours at 37°C and subsequently harvested with physiological saline. Nutrient agar plate counts of dilutions of the harvested suspension established the number of organisms per milliliter. The suspension was heat-treated at 60°C for 30 minutes and concentrated by centrifugation to approximately 10×10^{10} spores per milliliter. A sterile solution of saponin in distilled water was added to give a final concentration of 1×10^{9} spores per milliliter in 0.5 per cent saponin.

Ten milliliters of the heat-treated suspension (1×10^{9} spores per milliliter) were injected subcutaneously into the cervical region of a horse at weekly intervals for three weeks.* One year after the initial vaccination series, the agar/gel precipitin titer was found to be negative; a single 10.0 milliliter boosted injection of the same concentration of spores produced a maximum recall-titer of 1:256 six weeks later.

One- to three-liter bleedings were obtained two to seven weeks after booster injection. Serum was removed from clotted blood, distributed in suitable ampules, and stored at -15°C. Serum with a titer of 1:64 or greater was considered adequate.

D. PREPARATION OF AGAR DIFFUSION TEST PLATES

The test medium was prepared according to a formula and method devised by Thorne.[4] Ten grams of monobasic potassium phosphate (KH_2PO_4), 9.0 grams of sodium chloride (NaCl), and 10.0 grams of Bacto-Noble Agar (Difco) were added to one liter of distilled water. The mixture was dissolved by heating and the pH adjusted to 7.3 at 38°C. The solution was sterilized after which 0.1 gram of thimerosal was added, then filtered through three or four layers of #1 Whatman filter paper on a Buchner-type funnel of at least 150-millimeter diameter and dispensed in 200-milliliter samples into eight-ounce prescription-type screw-cap bottles for storage in the refrigerator at 4°C.

When needed, 12.0-milliliter portions of the stored medium were dispensed into flat, thick-bottomed Pyrex (Corning #3162) Petri dishes and allowed to solidify for 24 hours at 4°C. Circular reservoirs were then made in three parallel rows by suitable cutters passed through a Plexiglas template.[1]

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* In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

The cutters were prepared from stainless steel tubing with the ends
accurately bevelled to reproduce the recommended well diameters of seven
millimeters or five millimeters. Agar plugs were aspirated with large-
bore capillary pipettes attached to a vacuum system.

Plates were stored upright in Petri dish cans at 4°C and used for
periods up to three weeks without adverse effect on the test.

E.   AGAR DIFFUSION READING LAMP

The reading lamp (Figure 1) was fabricated from a cylindrical fiber
drum approximately one foot in diameter, eight inches in height, and
with a central hole in the lid of a diameter slightly less than that of
a Petri dish; other stable materials of similar dimensions could be used.
A wire test-tube basket, ten inches in diameter, was centered and fixed
to the bottom of the drum. The basket, the interior surfaces of the
drum and the undersurface of the lid were painted with flat black paint.
An eight-inch Circline bulb was mounted within the wire basket with wire
clips and fixed in a central position, one inch from the basket base. A
circular shield of rigid, black material (in the illustration, cardboard,
painted black, was used) was constructed with a diameter adequate to
block direct light rays from all but the outer circumferential half of
the fluorescent bulb. This assembly, along with suitable electric switch
and a trigger ballast (CE CAT #89G322, 1958), was affixed to a 12- by
24-inch platform of 3/4-inch plywood.

F.   "BOX" TITRATION OF ANTIGEN AND ANTIBODY SYSTEM

"Box" titration of anthrax antigen versus the anthrax hyperimmune serum
was set up in agar diffusion plates. Five serial twofold dilutions of the
antigen and of the antisera were prepared, using physiological saline as the
diluent and beginning with 0.5 milliliter of the antigen and 0.2 milliliter
of the antiserum. To each dilution an equivalent volume of physiological
saline was added to give final dilutions ranging from 1:2 to 1:32. The
center row of reservoirs (approximately 0.025 milliliter volume per
reservoir) was filled with one of the prepared serum dilutions, one plate
being used for each dilution; plates were incubated at 37°C for two hours
to allow prediffusion of the anthrax antibody so that the final antigen
antibody reaction occurred equidistant from the center to the outer rows
of reservoirs. Following incubation, each of the wells (approximately 0.07
milliliter volume per well) in the two outer rows was filled with the
antigen dilutions in sequence so that one row exactly duplicated the
opposite.

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Figure 1. Visualization Apparatus.
A. Top view demonstrating interior
construction and placement of
wire basket, Circline bulb and
cardboard blocking shield (not
in working position).
(FD Neg C-4797)
B. Exterior view of finished
product exhibiting centered
hole in the lid; (a Plexiglas
template with stainless steel
cutters is shown on the lower
right corner of the table).
(FD Neg C-4796)

Plates were observed with the aid of the visual apparatus previously described after remaining at 23°C to 28°C for 18 to 24 hours.

The end point was determined as that combination of the highest dilution of antigen and antibody that produced a visible line of precipitate equidistant between the center and outer rows of wells. This initial titration was essential to the establishment of maximum sensitivity and consistent reproducibility of subsequent tests with unknown sera. The end-point reading was arbitrarily assumed to represent a "minimum reacting dilution," or MRD_a, of antigen and a "minimum reacting dilution," or MRD_s, of antibody. This is illustrated in Table I, wherein the "MRD_a" of antigen was established as a 1:16 dilution and the "MRD_s" of antibody as a 1:2 dilution.

TABLE I. ANTHRAX ANTIGEN AGAR GEL BOX TITRATION
(MRD_a-1:16, MRD_s-1:2)

Antiserum Dilution	Antigen Dilution				
	1:2	1:4	1:8	1:16	1:32
1:1	+	+	+	±	±
1:2	+	+	+	+	-
1:4	+	±	±	±	-
1:8	-	-	-	-	-
1:16	-	-	-	-	-

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G. SERUM TITRATIONS

The inhibition, or indirect method, was used in titrating sera. Serial twofold dilutions of 0.2 milliliter of unknown serum were made in physiological saline. To each dilution, 0.2 milliliter of a twofold concentration (1:8) of the previously titered antigen (1:16) was added, the final mixture thus containing an antigen "MRD_a" plus unknown serum dilutions ranging from 1:2 to 1:32. The mixtures were hand-shaken for 30 seconds and incubated in a 37°C water bath for one-half hour to permit antigen/antibody binding to proceed to completion, rather than being added immediately to their respective outer wells as in the Thorne and Belton technique.

Each of the wells in the center row of the agar diffusion Petri dishes was filled with a 1:2 dilution (MRD_s) of known anti-anthrax horse serum and the plates were incubated at 37°C for two hours instead of using the Thorne-Belton technique, which employs concentrated anti-anthrax horse serum, incubated in the center wells at 2°C for 18 to 24 hours. Conclusion of incubation periods for the plates and the antigen/antibody mixtures was timed to coincide and permit immediate accomplishment of the final step.

Outer rows of wells were filled sequentially and in duplicate with the incubated antigen/antibody mixtures; thus, each well in one outer row contained the same mixture as the corresponding well in the opposite outer row. The end point was determined after plates remained at temperatures of 23° to 28°C for 18 to 24 hours, instead of a preliminary 24-hour reading with a final 48-hour reading according to the Thorne and Belton[1] technique, and was that dilution of the unknown serum that completely inhibited the formation of a visible line of precipitate (Figure 2).

Controls, subjected to the same test procedures, consisted of dilutions of the antigen alone in physiological saline and combinations of the predetermined "MRD_a" of antigen with known negative and positive anti-anthrax sera.

This modified method therefore required 20 to 26 hours for completion in contrast to the Thorne and Belton method, which required 58 to 72 hours.
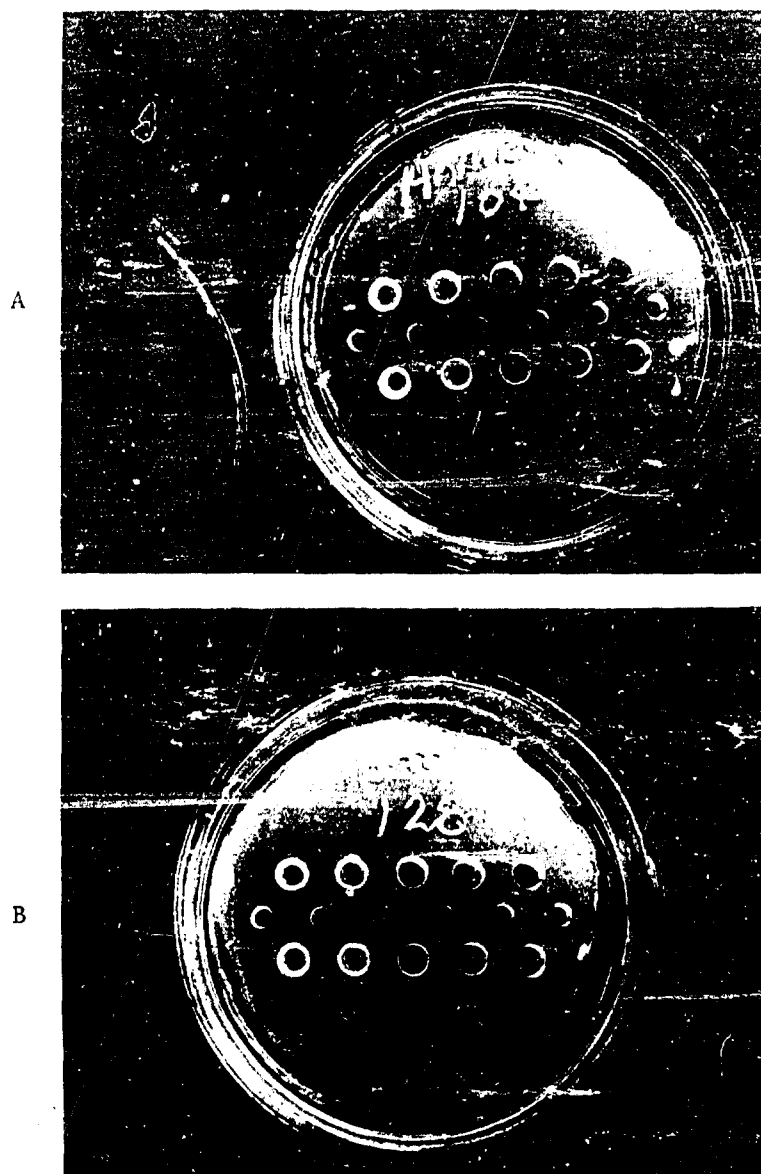
Figure 2. Serum Titrations by Indirect Technique.
  A. Negative serum: Note undulating lines of
      visible precipitate between center and
      outer rows of wells indicating absence of
      binding of MRD_a.
  B. Positive serum: Note absence of visible
      line of precipitate between first three
      wells on the left of center and outer
      rows, indicating complete binding of
      MRD_a; end point is 1:8 dilution of serum.

III. RESULTS

A comparison of the sensitivity of the modified indirect technique, incorporating the "box" titration concept, with the earlier method devised by Thorne and Belton[1] was conducted initially on 39 sera from individuals vaccinated with the anthrax protective antigen developed by Wright, Green and Kanode.[5] Agar-gel precipitin titers had been determined on these specimens by Thorne and Belton's[1] indirect technique; eight of the sera were negative and the remainder ranged in titer from 1:2 to 1:32. After storage for eight months at -15°C, these same sera were retitrated by the modified technique; resultant titers essentially duplicated the distribution obtained earlier.

A second group of 636 human anti-anthrax sera titered, stored and retitered in the same manner as the initial 39 specimens mentioned above, again resulted in a titer distribution with the modified technique that was in even closer agreement with results by the earlier method.

Reproducibility of results using the modified indirect technique exclusively was demonstrated by titration of 205 anti-anthrax sera and subsequent reevaluation by the same technicians after specimens had been stored at -15°C for three to five months. Ninety-four per cent of the titers were unchanged or varied only twofold, the twofold variations being considered within the limits of technical error; the remaining six per cent showed no more than a fourfold variance (Figure 3).

As a further evaluation of reproducibility, 56 "unknown" sera, previously titered by Mr. Frank D. Belton at the Microbiological Research Establishment Laboratories in Porton, England, by the original indirect procedure, were retitered by the newer technique. Again, 94 per cent of the results were either unchanged or varied only a single twofold dilution (Figure 4).

The superior sensitivity of the indirect technique as compared with the direct or double diffusion procedure was conclusively demonstrated with a group of 39 human anti-anthrax sera (Figure 5). The direct procedure involves a direct diffusion of known anthrax antigen against unknown serum dilutions in agar-gel plates. The observance of a line of precipitate indicates an anthrax-positive reaction in the serum dilution. The graphic pattern of titer distribution with the indirect method is suggestive of a mirror image of the titer distribution employing the direct technique. Fifty per cent fewer negative sera were obtained with the indirect technique.
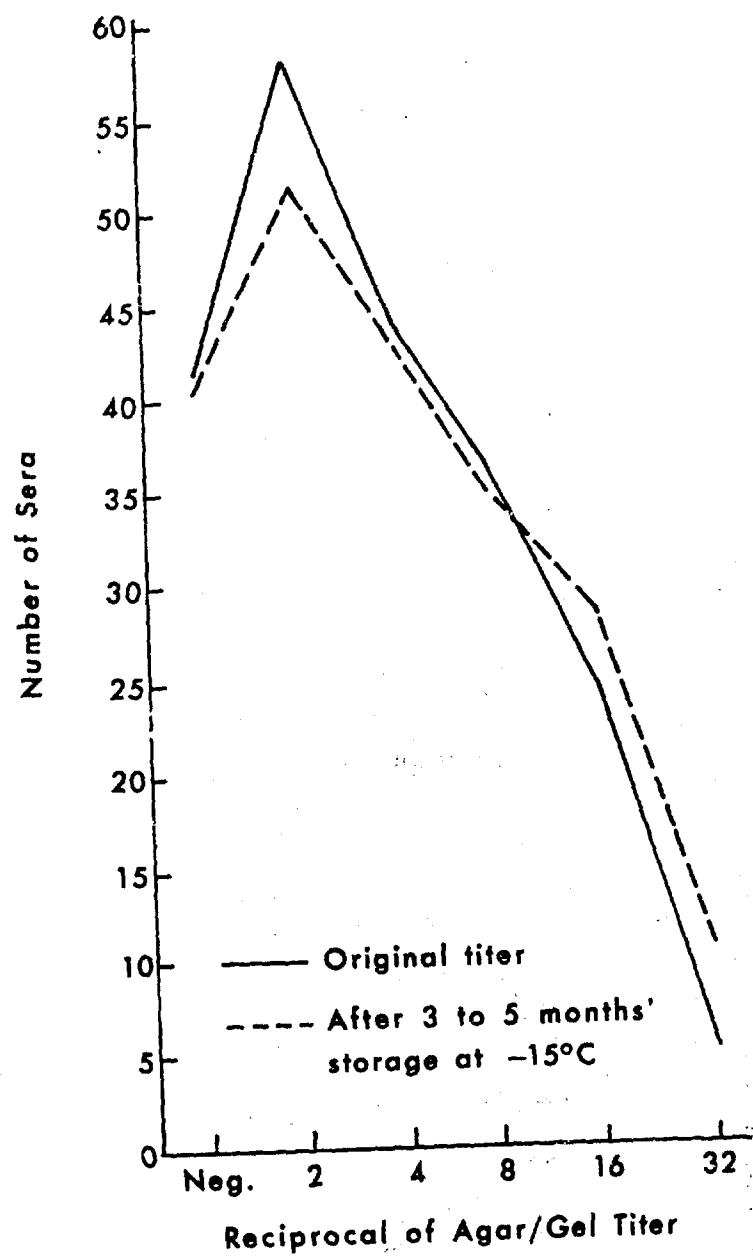
Figure 3.   Reproducibility of 205 Anti-Anthrax
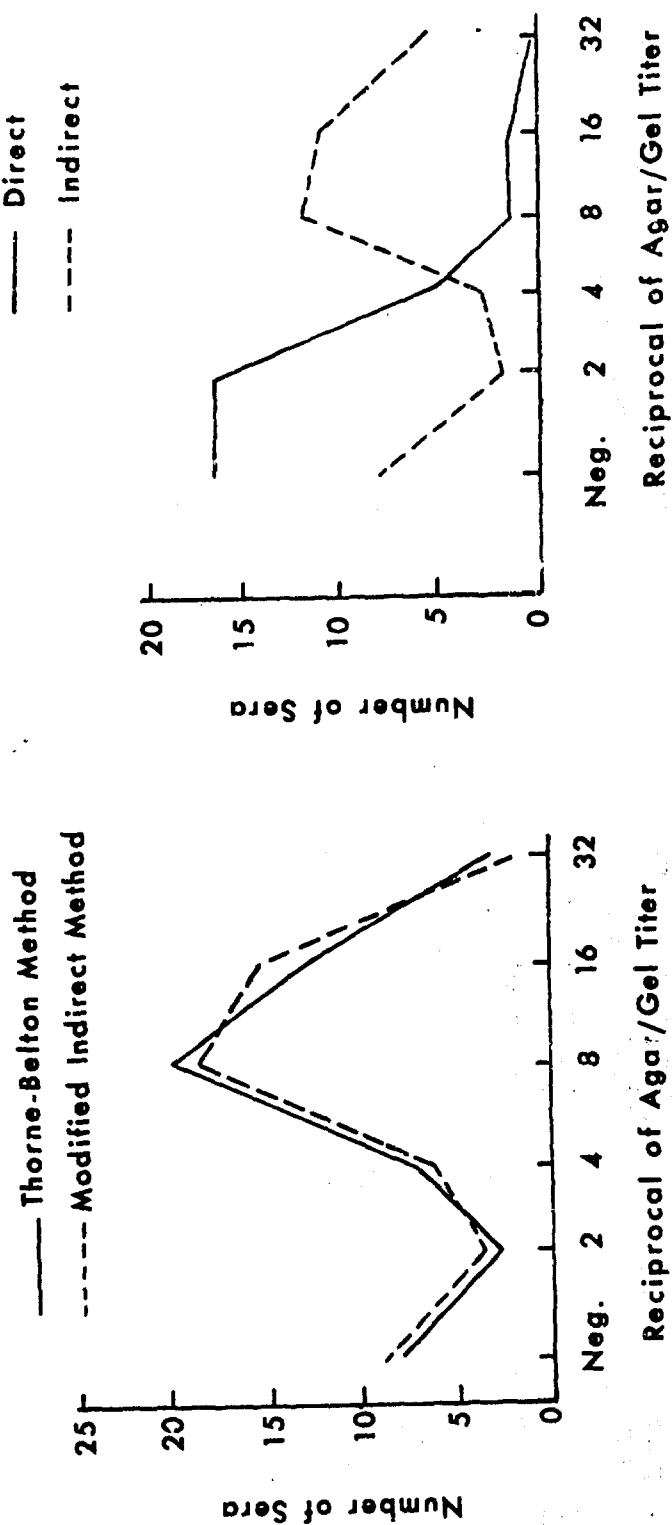Sera Titers Employing the Modified
Indirect Method.

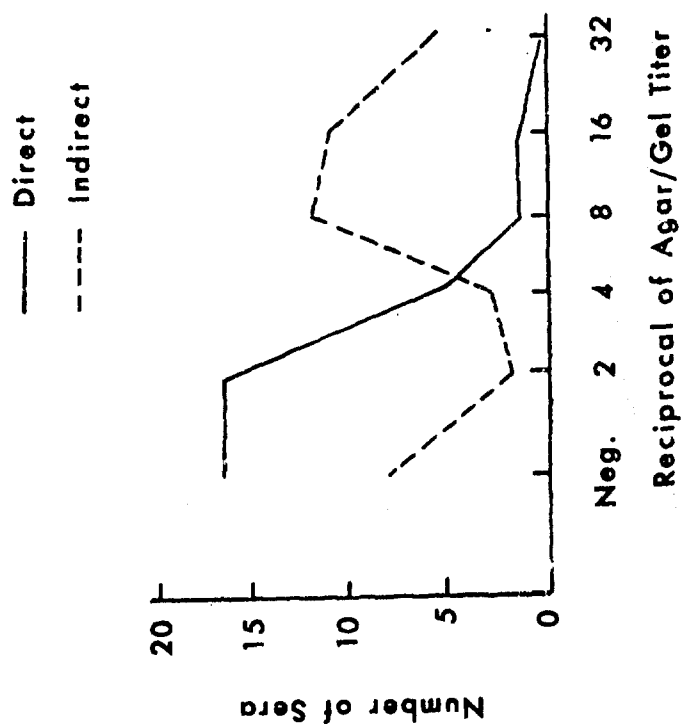Figure 4. Comparison of the Modified Indirect and Thorne-Belton Methods, Employing 56 "Unknown" Sera.

Figure 5. Comparison of Titer Distribution Employing the Direct Versus Indirect Techniques on 39 Sera From Individuals Vaccinated with the Anchrax Protective Antigen.

Thorne[6] was able to produce culture filtrate antigens from <u>Bacillus</u> <u>cereus</u> but not from <u>Bacillus</u> <u>subtilis</u> or <u>B</u>. <u>megatherium</u> grown under the same conditions for anthrax antigen production. However, these antigens were very different from those produced by the anthrax bacilli, and anthrax antiserum did not inhibit the action of these antigens in mice or guinea pigs as shown by Thorne[8] and Molnar.[7]

In our laboratory, we cultured strains 246, 4342, 7064, 7483, 9139, 9592, and 10376 of <u>B</u>. <u>cereus</u>, strains 6537, 7972, 9858, and 10783 of <u>B</u>. <u>subtilis</u>, and strains 6458, 6459, 7703, and 11561 of <u>B</u>. <u>megatherium</u> under the same conditions as cited for the production of anthrax antigen. <u>Bacillus</u> <u>cereus</u> strains 246, 4342, 7064, 7483, and 9139 and <u>B</u>. <u>megatherium</u> strain 6458 produced culture filtrate antigens, but our findings confirm those of Thorne[8] in that the antigens were not the same as those produced by the anthrax bacillus.

As a further check on specificity of the modified indirect technique, several human and animal sera of significant titer were selected for each of the following diseases: brucellosis, influenza, listeriosis, plague, poliomyelitis, psittacosis, Q fever, and Rocky Mountain spotted fever. When subjected to the same procedure incorporating the anthrax antigen, these sera produced a visible line of anthrax precipitate indicating no cross-reactivity or inhibition of the anthrax precipitation reaction in the agar/gel plates.

IV. DISCUSSION

The agar diffusion (inhibition) method described by Thorne and Belton,[1] although a sensitive and simple method for assaying antigen concentrations in culture filtrates, did not prove satisfactory as a routine serological procedure for titrating antisera from individuals vaccinated with the anthrax protective antigen. Visualization of lines of precipitate, essential to accurate determination of titer end points, was extremely difficult and often impossible, especially for the technician unfamiliar with the re-action.

To obviate this handicap, incorporation of the concept of preliminary "box" titration of antigen and antibody, modifications of methods for com-bining reagents, variation of reactant incubation periods, and development of an improved visualization apparatus eventually evolved a procedure that reduced by approximately one-third the time required for the test and greatly contributed to the ease of end-point determinations, even by the novice, without detracting from the simplicity of the test.

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Agreement of the modified indirect technique has been amply demonstrated by multiple comparative evaluations with the earlier Thorne and Belton[1] technique, utilizing sera from varied human and animal sources. Thorne and Belton[1] and Strange and Thorne[3] have already established the anthrax antigen/antibody reaction as a single-line phenomenon according to the conditions of their test procedure. Our results apparently further confirm this observation.

When applying this serological test to sera from rabbits* vaccinated with various anthrax antigen preparations and subsequently challenged with virulent anthrax spores (data to be published), it was ascertained that an even higher degree of sensitivity could be attained by mixing the "MRD_a" with each of the serial dilutions of unknown or anthrax antiserum, rather than the heretofore recommended twofold concentration of the "MRD_a." End points were still readily determined at 24 hours and at least a twofold rise in titer was attained by this procedure. It can therefore be assumed that an equivalent increase in sensitivity might be expected irrespective of source of antiserum. However, for purposes of this paper, the antigen in the greater concentration sufficed to validate the sensitivity comparison of the new procedure with the Thorne and Belton[1] technique.

Initial explorations of this technique in evaluating antibody response in botulism, coccidioidomycosis, histoplasmosis, influenza, plague, poliomyelitis, psittacosis, Q fever, staphylococci food poisoning, and variola, have been promising. Definition of the conditions for conducting the test and the expected results for each of the aforesaid diseases are nearing completion (to be published).

V. SUMMARY

1. An improved and modified agar/gel precipitin technique for evaluation of anthrax antibodies is presented.

2. The procedure has been demonstrated to be less time-consuming and retains satisfactory reproducibility, simplicity, specificity, and sensitivity when compared with the previously utilized technique.

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* In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

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